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Canavanine in the Leguminosae

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Canavanine (α-amino-δ-guanidoxybutyric acid) was originally discovered in three closely related species of *Canavalia* (Kitagawa & Tomiyama, 1929; Kitagawa, 1937; Damodaran & Narayanan, 1939). Subsequently it was identified in the seeds of 17 other leguminous plants and isolated from three of them (Fearon & Bell, 1955; Bell, 1958). Evidence is now given for the presence of canavanine in the seeds of other leguminous plants.

Bell (1958) suggested that canavanine stored in leguminous seeds provides a readily available supply of nitrogen for the developing embryo. It is now shown that the concentration of canavanine in the seeds of *Medicago sativa* does in fact fall rapidly during germination.

The apparent restriction of canavanine to the Leguminosae (Fearon & Bell, 1955) and its occurrence in 31 plants of this group suggested that its biosynthesis might be directly or indirectly associated with the presence of nitrogen-fixing bacteria in the root nodules of these plants. Canavanine has therefore been tested for in both the cells and growth media of bacteria capable of fixing nitrogen whilst living in the free state and also of bacteria known to fix nitrogen while living in symbiotic association with leguminous plants. Plants of *M. sativa* and *Trifolium pratense* grown in the presence and absence of their respective rhizobia, and the seeds of certain non-leguminous plants reported to fix nitrogen, have been tested for canavanine.

EXPERIMENTAL

Pentacyanoammonioferrate reagent and phosphate buffer (pH 7). These were prepared as described by Bell (1958).

Qualitative analysis of seeds

Preparation of seed extracts. Finely ground seed (0·1–0·3 g.) was stirred with 1 ml. of 0·1 N-HCl at room temperature and kept for 18 hr. The suspension was then neutralized with 1 ml. of 0·1 N-NaOH and the supernatant was used for paper chromatography and ionophoresis.

Chromatography. The solvents used were phenol–water (4:1, w/v), butanol–pyridine–acetic acid–water (4:1:1:2, by vol.) and solvent F of Reio (1958) [methyl ethyl ketone–acetone–water–formic acid (80:4:12:2, by vol.)]. Chromatograms were run on Whatman no. 1 paper for 24 hr. in the first two solvents and for 5 hr. in the third. The chromatograms run in the second solvent were sprayed with phosphate buffer and redried in air to remove the last traces of pyridine (Bell, 1958). All chromatograms were sprayed with pentacyanoammonioferrate (PCAF) reagent, which gives a characteristic magenta spot with canavanine.

Ionophoresis. Ionophoresis was carried out at pH 7 as described by Bell (1958), and the dried papers were sprayed with PCAF reagent.

PCAF reaction negative. Extracts of the seeds of the following plants gave no colour with PCAF.

Leguminosae: *Baptisia australis, Cercis siliquastrum, Clitoria ternatea, Cytisus albus, C. praecox, C. scroparius, C. sessilifolius, Dolichos lablab, Genista hispanica. G. sagittalis, G. tinctoria, Glycine soja, Laburnum anagyroides, Lespedeza buergeri, Lupinus arboreus, L. littoralis, L. nootkatensis, L. polyphyllus, L. subcarnosus, Melilotus alba, Mimosa pudica, Onobrychis caput-galli, Petteria ramentacea, Spartium junceum, Tetragonolobus purpureus, Vicia angustifolia, V. lutea, V. sativa, Vigna cylindrica.*

Coriariaceae: *Coriaria terminalis, C. thymaefolia.*

Elaeagnaceae: *Elaeagnus angustifolia, E. pungens reflexa, E. umbellata, Hippophae rhamnoides.*

PCAF reaction positive. A compound giving a magenta reaction with PCAF at pH 7 and occupying the same position as authentic canavanine on the chromatography and ionophoresis papers was found in the seeds of the following Leguminosae: *Astragalus alopecuroides, A. cicer, Caragana arborescens, Colutea media, Lotus uliginosus, Medicago falcata, M. lupulina, Ononis rotundifola, Securigera securidaca, Trifolium pratense, Vicia hirsuta.*

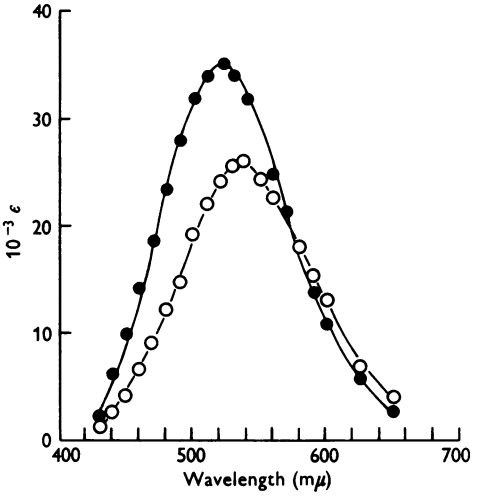

Fig. 1. Absorption spectra of canavanine–PCAF (●) and deaminocanavanine–PCAF (○). Solutions were prepared as described in the text.

Absorption spectra

Canavanine–PCAF. To 0·25 mg. of canavanine in 10·5 ml. of buffer solution was added 0·1 ml. of PCAF reagent. The solution was kept for 40 min., after which time the colour had fully developed. The absorption spectrum was determined in a Unicam SP. 500 spectrophotometer with a mixture of 10·5 ml. of buffer and 0·1 ml. of PCAF reagent as 'blank'. The curve obtained (Fig. 1) showed a single peak with a maximum at 522 mμ.

Deaminocanavanine–PCAF. To 0·32 mg. of deaminocanavanine (hexahydro-3-imino-1-oxa-2:4-diazepine-5-carboxylic acid) in 10·5 ml. of buffer solution was added 0·1 ml. of PCAF reagent. The solution was kept in the dark for 2 hr. The deaminocanavanine colour developed more slowly than the canavanine colour and was less stable in daylight. This compound showed a single absorption maximum (Fig. 1) at 536 mμ. Deaminocanavanine is not known to occur naturally in the Leguminosae but is formed by the decomposition of canavanine in aqueous solution even at 30°.

Estimation of canavanine

For the determination of canavanine in the individual seeds of *M. sativa* the sensitivity of the colorimetric method of Fearon & Bell (1955) was improved by using a spectrophotometer and restricting measurements to the absorption maximum. By these means a concentration–extinction curve was prepared with canavanine standards of approximately one-tenth of the original concentration. The smaller cell size of the instrument also contributed to an overall increase of some 30-fold in sensitivity. A linear curve passing through the origin was obtained up to a concentration of 0·14 m-mole/l., at which the extinction in a 1 cm. light path was 0·52.

Disappearance of canavanine from seeds of Medicago sativa *during germination*

Seeds of the same batch were used for all experiments.

Canavanine content of seeds. Seeds (20, weight range 2·0–3·2 mg.) of *M. sativa* were weighed separately, ground and triturated with 3 ml. of buffer solution. To each suspension was added 0·1 ml. of PCAF reagent; after 2 hr. the suspensions were filtered and the extinction of each filtrate was determined at 522 mμ. The canavanine content was between 0·5 and 1·4 % of the weight of the seeds examined. Bell (1958) found 1·46 % for a batch of seed which had been ground, mixed and dried at 110° for 20 hr. before analysis. Measurements made after adding known weights of canavanine confirmed that 0·001 mg. of the amino acid could be determined in the seed extracts.

Germination of seeds on sand. Seeds of *M. sativa*, of the same weight range as those analysed, were surface-sterilized with concentrated sulphuric acid and, after washing 10 times with sterile water, were allowed to germinate in individual tubes. One group of seeds was supported on 'slopes' of fine sand covered at the lower end with water. A second group was grown on sand watered with a solution of mixed salts containing (%): $CaHPO_4$, 0·1; K_2HPO_4, 0·02; NaCl, 0·02; $MgSO_4,7H_2O$, 0·02; $FeCl_3$, 0·01; and (p.p.m.) $Na_2MoO_4,2H_2O$, 2·5; $CuSO_4,5H_2O$, 3·9; $MnSO_4$, $4H_2O$, 9·3; $ZnSO_4,7H_2O$, 4·4; H_3PO_3, 5·6. Ammonium sulphate equivalent to 2 mg. of N/seed was also added to each tube of this group. All tubes were sterilized in the autoclave before the seeds were introduced. After developing on the sand 'slopes' for 7 days, 40 seedlings which had been supplied with the mixed-salts solution and $(NH_4)_2SO_4$ were removed from their tubes. A further 40 seedlings were removed after 14 days' growth. The sand in each tube was filtered and washed free of adhering liquid with buffer solution. The combined filtrate and washings were made up to 3 ml. To each tube was added 0·1 ml. of PCAF reagent and 40 min. was allowed for colour development. No canavanine was detected in any of the 80 tubes.

Each of the seedlings was crushed and triturated with 3 ml. of buffer solutions; 0·1 ml. of PCAF reagent was added to each suspension and 40 min. allowed for colour development. Of the seedlings grown for 7 days, six of those supplied with water and seven of those supplied with salts solution gave a positive reaction for canavanine. Of those grown for 14 days, four supplied with water and two supplied with salts solution gave a positive reaction. No colour developed in the remaining tubes on further keeping.

The addition of canavanine (one ground ungerminated seed) to each of the seedling extracts which gave a negative reaction and to each of the tubes of media produced a positive result. The sensitivity of the PCAF test in the presence of the crushed seedlings was determined by adding canavanine to the extracts. An amount of 0·004 mg. of canavanine was readily detectable.

Growth of seedlings on an agar medium. Sterile seeds of *M. sativa* and *T. pratense* were sown individually in test tubes on 'slopes' containing 1·2 % of agar (Difco Bacto) and inorganic salts in the same proportions as in the mixed-salts solution described. The medium was adjusted to pH 6·6–6·7 with 0·1 N-NaOH. The seeds were divided into four groups each containing 20 of each species. To one group was added $NaNO_3$ (2 mg. of N/tube) and to a second group was added an equivalent weight of $(NH_4)_2SO_4$. The seeds of *M. sativa* in the third group were innoculated with *Rhizobium meliloti* (Rothamsted AH_I) and those of *T. pratense* with *Rhizobium trifolii* (Rothamsted ClF). The remaining seeds were retained as controls. Sterile water and a diluted mixed-salts solution were added to the plants as required during growth. Half the plants were analysed after 6 weeks' and the remainder after 10 weeks' growth. The whole plants were extracted with 3 ml. of buffer solution and treated with 1 ml. of PCAF reagent. No canavanine was detected in any group after 6 weeks' growth. Positive results were obtained only with 10-week plants to which $(NH_4)_2SO_4$ or $NaNO_3$ had been added. These results are in agreement with the finding that both nodulated and non-nodulated plants of *M. sativa* grown for 17 weeks with added NH_4NO_3 under field conditions gave a positive test for canavanine.

Qualitative analysis of bacterial extracts and media

Azotobacter vinelandii and *A. chroococcum* were grown in a liquid medium containing (%); mannitol, 2·0; K_2HPO_4, 0·3; $CaCO_3$, 0·1; NaCl, 0·1; $FeCl_3$, 0·001; $MgSO_4,7H_2O$, 0·001; and (p.p.m.) $Na_2MoO_4,2H_2O$, 1·0. The pH was adjusted to 7·2 with 0·1 N-NaOH. *Rhizobium meliloti* was grown in a medium containing (%): sucrose, 0·5; yeast extract (Yeastrel), 0·5; K_2HPO_4, 0·5; $MgSO_4,7H_2O$, 0·1; $FeCl_3$, 0·001; Na_2MoO_4, $2H_2O$, 0·0001; the pH was adjusted to 7·0 with 0·1 N-NaOH. After time had been allowed for the

colonies to establish themselves, 7, 10 and 21 days respectively, the bacteria were centrifuged and the culture medium was decanted. Part of each sediment of bacterial cells (0·5–1·0 ml.) was ground with fine sand and stirred with 2–3 ml. of 0·1 N-HCl. After 1 hr. the supernatant liquid and the decanted culture medium were examined by paper chromatography. No canavanine was detected in either the cell extracts or the culture media.

SUMMARY

1. Canavanine has been detected in the seeds of 11 species of leguminous plants.

2. The sensitivity of the method for the colorimetric determination of canavanine has been increased by using a spectrophotometer at 522 mμ.

3. Canavanine stored in seeds of *Medicago sativa* is metabolized by the seedlings during germination and growth.

4. The biosynthesis of canavanine by *Medicago sativa* and *Trifolium pratense* is not dependent upon the presence of nitrogen-fixing bacteria in the root nodules of the plants.

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Spectral-Absorption Coefficients of some Porphyrins in the Soret-Band Region

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All porphyrins exhibit intense fluorescence when illuminated by light of appropriate wavelength and this property has been much used in the past for their quantitative determination. The intensity of the fluorescence varies with pH (Fink & Hoerburger, 1933, 1935; Rimington, 1943; Jope & O'Brien, 1945), however, and is also much affected by impurities and even by the presence of inorganic ions. Notwithstanding the use of a reference standard for each determination, errors may be large and unpredictable.

With the advent of modern photoelectric spectrophotometers, it has become possible to exploit for quantitative measurement another characteristic physical property of porphyrins, namely their intense spectral absorption at about 400 mμ. This band, known as the Soret band, is intense and narrow and is most well developed in acid solution; its position and magnitude differ for each porphyrin. Both parameters are influenced by pH (cf. Jope & O'Brien, 1945) but conditions for measurement are easily standardized and non-absorbing impurities are without effect. For measurement in the presence of substances which absorb light, a correction formula has been devised by Rimington & Sveinsson (1950). This is generally applicable so long as the absorption of the impurities remains approximately linear over a narrow spectral range on either side of the Soret maximum of the porphyrin in question.

The porphyrins in acid solutions obey Beer's law up to an extinction (optical density) of about 1·0, and spectrophotometry is thus the method of choice for their determination. It is necessary, however, to know the molecular extinction coefficients (ϵ) of the pure materials under the conditions which are to be applied for their measurement. Unfortunately the preparation of porphyrins or their methyl esters in the pure state is difficult; melting points are unsatisfactory criteria of purity and it is thus not surprising that widely differing values for ϵ or $E^{1\%}_{1\,cm.}$ are to be found in the literature. The situation has been reviewed briefly by With (1955). The purpose of the present paper is to present the values found by examination of a number of carefully purified porphyrins and so to provide data for accurate spectrophotometry. Measurements have been made upon porphin and aetioporphyrin I for comparison with the naturally occurring porphyrins.